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Preparation, Characterization, and Efficacy of Cell Wall
and Ribosomal Vaccines from Legionella pneumophila

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Interim report

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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achieved by both i.p. and s.c. vaccination with 5 µg of cell wall protein. Vaccination i.p. with 50 µg of ribosomal antigen from the Philadelphia L-1 strain, resulted in 50% protection of mice against lethal challenge. Cell wall preparations, on the other hand, afforded full protection. Neither ribosomes or cell walls from Philadelphia L-1 protected mice when administered s.c. Microagglutination titers with Washington strain antigen, unlike those with Philadelphia L-1 antigen, correlated with protective response. Immunoprecipitin analyses indicated greater cross-reactivity between strains in those antisera produced with cell wall or ribosomal preparations from the Washington strain than in those produced with the Philadelphia L-1 strain preparations.

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ABSTRACT

The Washington and Philadelphia L-1 strains of Legionella pneumophila were mechanically disrupted and cell wall and ribosomal subunits isolated by differential centrifugation. Complete protection of AKR/J mice against challenge with the virulent Washington strain was obtained with intraperitoneal (i.p.) vaccination using as little as 5 µg of protein from ribosomes of the Washington strain. A higher concentration of ribosomal protein was required to achieve full protection when vaccination was by the subcutaneous (s.c.) route. Complete protection with cell wall preparations from the Washington strain was achieved by both i.p. and s.c. vaccination with 5 µg of cell wall protein. Vaccination i.p. with 50 µg of ribosomal antigen from the Philadelphia L-1 strain, resulted in 50% protection of mice against lethal challenge. Cell wall preparations, on the other hand, afforded full protection. Neither ribosomes or cell walls from Philadelphia L-1 protected mice when administered s.c. Microagglutination titers with Washington strain antigen, unlike those with Philadelphia L-1 antigen, correlated with protective response. Immunoprecipitin analyses indicated greater cross-reactivity between strains in those antisera produced with cell wall or ribosomal preparations from the Washington strain than in those produced with the Philadelphia L-1 strain preparations.

The epidemiology and clinical manifestations of Legionnaires' disease have been extensively defined (13, 22, 29, 30); however, the immunobiology of the disease, the roles of cellular and humoral immunity and the immunological response to different antigens have been poorly categorized. The identification and characterization of antigens which may be involved in eliciting immunological protection is required for the development of effective vaccines against this disease. In this regard, the AKR/J mouse is an appropriate model for the study of immunological protection against infection with Legionella pneumophila, the causative agent of Legionnaires' disease (15). Although the guinea pig is the recommended animal model for Legionnaires' disease (22), the AKR/J mouse was selected because it is susceptible to live challenges as well as the toxic moiety isolated from Washington strain organisms (15). Mice either passively immunized with goat antibodies to an attenuated strain or actively immunized with a soluble antigen are fully protected from a lethal challenge with virulent L. pneumophila.

In the present study, we examined the immunological protection afforded to AKR/J mice by immunization with preparations of killed whole organisms or subcellular fractions prepared from either the virulent Washington or avirulent Philadelphia strains. The preparation and physical and biochemical characterization of cell wall and ribosomal subunits from L. pneumophila are described.

MATERIALS AND METHODS

Animals. Female AKR/J mice (Jackson Memorial Laboratories, Bar Harbor, Me.), weighing 23 to 26 g, were used at 8 to 10 weeks of age.

Bacterial cultures. Modified Mueller-Hinton agar (10) slant cultures of Legionella pneumophila serotype 1 strains Washington and Philadelphia L-1 were obtained from the Centers for Disease Control, Atlanta, Ga., grown for 10 days and harvested in tryptose-saline (pH 7). Aliquots were stored at -70°C in tryptose-saline containing 50% fetal calf serum. To prepare working cultures, Mueller-Hinton agar plates were inoculated with a 1:100 dilution of the frozen stock and incubated at 37°C in 2.5% CO_2 . Cultures were harvested after 4 days by washing the bacterial growth from the surface of the agar plates with 0.15 M NaCl. The bacteria were collected by centrifugation at $2500 \times g$ for 30 min at 4°C and washed twice with 0.15 M NaCl. Washed pellets of the Washington strain intended for animal challenge were resuspended in tryptose-saline at a concentration of approximately 3×10^{10} bacteria/ml and aliquots were frozen at -70°C . Pellets of the Washington and Philadelphia L-1 strains harvested for physical and biochemical fractionation analyses were weighed and stored at -20°C . Purity of the harvest suspensions was assured by plating on blood agar media.

Isolation of cell fractions. Unless otherwise indicated, the phosphate buffer used for fractionation procedures contained 0.2 mM potassium phosphate (pH 7) and 30 mM MgCl_2 . Frozen bacterial pellets were suspended in four volumes (w/v) of phosphate buffer and 5 volumes (w/w) glass beads (0.10 to 0.11 mm diameter, B. Braun Instruments, San Francisco, Calif.) and homogenized in a Braun cell homogenizer Model MSK at 4000 rpm for 5 min at 30-sec intervals. After each 30-sec disruption period

the 70-ml stainless steel cup was cooled at -70°C for 5 min. The resulting homogenate was subjected to differential centrifugation (Fig. 1). The crude cell wall fraction (Pellet III) was sonically treated with a Fisher Sonic Dismembrator Model 300 for 3 min at 60% setting to ensure lysis. Pellet IV was resuspended in 0.6 M phosphate buffer (pH 7) and incubated for 18 h at room temperature with 10 mg RNase/ml suspension (Bovine ribonuclease A, Sigma Chemical Co., St. Louis, Mo.) to digest residual RNA. Chloroform was added to prevent growth of microbial contaminants during incubation. Aliquots from each pellet and supernatant were held at -20°C for biochemical assays and determination of total recoveries. Additional frozen aliquots from the cell wall fraction (Pellet IV A) and the ribosomal fraction (Pellet V) were reserved for immunological studies.

Biochemical analyses. Protein was determined by measuring the binding of Coomassie brilliant blue G-250 (BioRad protein assay, BioRad Laboratories, Richmond, Calif.) (1, 3). Lyophilized bovine plasma gamma globulin was used as a reference standard (BioRad). A phenol-sulfuric acid method (8) was used to determine carbohydrate (CHO) concentrations, the Schmidt-Thannhauser procedure (26) as modified by Fleck and Munro (2, 11), for RNA assay, the Burton procedure (4), for DNA, and the method of Karkhanis et al. (20) for evaluating 2-keto-3-deoxyoctonic acid (KDO). The corresponding reference standards were: sucrose, yeast RNA (CalBiochem-Behring Corp., San Diego, Calif.), calf thymus DNA (Sigma), and KDO (Sigma). Optical density measurements for the BioRad assay were recorded on a Gilford Stassar II spectrophotometer; all others were recorded on a Beckman DB-G spectrophotometer.

Isopycnic centrifugation on sucrose gradients. Analyses by equilibration density centrifugation on sucrose gradients were performed

in Quick-seal tubes (Beckman, 25 x 89 mm, Beckman Instruments, Inc., Palo Alto, Calif.) after successively layering a 5-ml cushion of 66% sucrose, 28 ml of a sucrose gradient extending linearly with respect to volume from 30 to 60%, and a known volume of cell wall sample suspended in water. Centrifugation was carried out in a Beckman L5-65 ultracentrifuge with a type Vti 50 horizontal rotor preset at 6000 ω^2 t at 40,000 rpm, and 18 fractions of 2 ml each were collected in tared tubes. Volumes were determined gravimetrically and the percent sucrose in each fraction measured with an Abbe refractometer (American Optical Corp., Buffalo, N.Y.). The turbidity of the sample and its fractions was determined spectrophotometrically at 540 nm. Distribution patterns of turbidity in the isopycnic gradients were normalized to permit averaging of results from different experiments (5).

Electron microscopy. The homogeneity of the final preparations of cell walls and ribosomes was assessed by electron microscopy. Samples were fixed in 2% glutaraldehyde for 2 h at room temperature, centrifuged, and resuspended in 0.1 M cacodylate sucrose buffer. Thin sections were stained with uranyl acetate-lead citrate and observed on a Hitachi HU-12 electron microscope.

Immunization and challenge of animals. Immunizing dosage of the ribosome or cell wall fraction preparations of the Washington and Philadelphia L-1 strains ranged from 0.05 to 5 μ g of protein. A primary injection of 0.2 ml subcutaneously (s.c.) or 1.0 ml intraperitoneally (i.p.) was administered to mice, repeated 28 days later as a booster injection. All mice were challenged i.p. 10 days later with a 1.0 ml dose containing 2×10^8 colony forming units (CFU) of the virulent Washington strain. Deaths were recorded daily for 15 days.

Production of antibodies. Antisera against killed whole organisms were produced in mice by i.p. injection with 1.0 ml of suspensions that were heated at 60°C for 60 min and contained the equivalent of 10^7 CFU. Antisera against Washington or Philadelphia L-1 cell fractions of cell walls or ribosomes were produced by i.p. injection with 50 µg of protein contained in 1.0 ml of each preparation. A booster injection of the same material was administered after 28 days and serum collected 10 to 11 days later. Heat-killed bacterial suspensions adjusted to an optical density of 0.660 at 490 nm were used as antigens for microagglutination tests.

Immunodiffusion. Double gel diffusion analysis of antigens was done in petri dishes layered with 10 ml of buffered agarose gel [0.5% agarose (Sea Kem), 0.04 M NaCl, 0.05 M borate buffer, pH 8.3 to 8.6]. A 80-µl volume of antiserum or antigen was added to appropriate wells. Maximal precipitin reactions were determined by titrating the antigens with the various antisera. After incubation for 2 days at 37°C in a humid atmosphere the plates were rinsed with three changes daily of 0.15 M NaCl for at least 3 days. Washed plates were treated with 2% acetic acid (v/v) for 5 min and then stained with 0.25% buffalo black and 0.025% Coomassie blue in 10% acetic acid for 15 min. After destaining the gel was photographed.

RESULTS

Efficacy of mechanical disruption. The effectiveness of mechanical lysis of a L. pneumophila suspension with the Braun homogenizer was established by comparison with a method of lysis by NaOH or sodium dodecyl sulfate (SDS). Optimal disruption of bacteria as measured by maximal clearing of the suspension and solubilization of protein was shown by the combined treatment of 0.5 N NaOH and 0.05% SDS.

The effect of mechanical disruption with the Braun homogenizer is shown in Figure 2. Turbidity was recorded at 420 nm on a spectrophotometer on samples diluted in distilled water. These samples were then centrifuged for 15 min at 4000 x g and the quantity of soluble protein in the supernatants were expressed as the percent of protein solubilized by treatment of the suspension with 0.5 N NaOH and 0.05% SDS.

Isolation of cell wall and ribosome fractions. Figure 3A confirms the observations that L. pneumophila is a rod-shaped bacterium with a bilaminar outer sheath presumed to consist of outer membrane and cell wall or peptidoglycan that enclose cytoplasmic ribosomes, nucleoplasm, and some vacuoles (6, 12). Treatment of L. pneumophila with the Braun homogenizer caused extensive disruption with release of cellular contents as indicated by empty and partially empty cells (Fig. 3B). Pellet II contained few disrupted cells and only small amounts of the total protein, CHO, DNA, and RNA (Table 1). Pellet III consisted predominantly of cell walls devoid of cytoplasmic particles (Fig. 3C). Incubation of cell walls with RNase without sonic treatment caused no noticeable difference or change in the fraction; however, aggregates appeared in the cell wall preparation following this treatment. A

preparation of sonically treated cell walls after incubation with RNase (Pellet IVA) shown in Fig. 3D consisted of intact cell walls, cell wall fragments, and cell wall aggregates.

To assess the degree of homogeneity of the cell wall preparation, crude cell walls (Pellet III) and the final cell wall fraction (Pellet IV A) were subjected to isopycnic ultracentrifugation in sucrose gradients. Both preparations gave a single band which had a relatively narrow equilibrium density distribution pattern with a mean density of 1.22 g/cm^2 (Fig. 4), and corresponded well with the value reported for L. pneumophila cell walls by Flesher et al. (12). The ribosomal fraction (Pellet V) was composed of dense cytoplasmic granules with no cell wall contamination (Fig. 5).

The percent distribution data (Table 1) revealed that the fractionation procedure was quite efficient in that the total recovery of measured biochemical constituents ranged from 73 to 95%. Equally significant was the 1:3 (0.289) ratio of RNA to protein in the ribosomal fraction. In the cell wall fraction (Pellet IV A) however, RNA contamination was less than 2 parts per 1000 of protein (0.0017).

Immunogenic properties of cell wall and ribosomal fractions.

Comparisons between the immunogenicity of Washington and Philadelphia L-1 cell wall and ribosome preparations are presented in Table 2. Optimal protection against a lethal dose of Washington strain organisms was afforded when animals received a booster dose 4 weeks after initial immunization. The median protective dose (PD_{50}) for vaccination and booster with cell walls from the Washington strain was $2.5 \mu\text{g}$ protein for the s.c. route and less than $0.05 \mu\text{g}$ for the i.p. route. In contrast, the PD_{50} for Washington strain ribosome preparations was 5 and $0.4 \mu\text{g}$

protein for the s.c. and i.p. routes, respectively. The corresponding PD_{50} values for Philadelphia L-1 vaccinees can be calculated only for the i.p. route of immunization. These values are 50 μ g protein for ribosome preparations and less than 5 μ g protein for cell wall material. Vaccinees receiving heat-killed whole-cell preparations from the Washington strain showed complete protection. However, similar Philadelphia L-1 whole-cell preparations conferred only 33% protection after challenge with the Washington strain (unpublished observations).

Microagglutination titers for mice immunized with 50 μ g ribosomal or cell wall protein of the Washington or Philadelphia L-1 preparations and bled 10 days after the booster are shown in Table 3. Serologic titers for normal AKR/J mice were less than 1:2 with either heated bacterial antigen. Immunization with Washington strain ribosomal or cell wall preparations evoked equivalent titers for both Washington and Philadelphia L-1 antigens. Although the protection afforded by either vaccine preparation was complete regardless of immunization route, microagglutination titers were markedly higher after i.p. immunization with cell walls. Immunization with Philadelphia L-1 strain ribosomal and cell wall preparations produced markedly lower titers against Washington antigen than against the homologous strain antigen.

The serologic response following immunization was measured by microagglutination assays in mice vaccinated twice at 28-day intervals (50 μ g protein in 1.0 ml i.p.) with Washington strain ribosome or cell wall preparations (Fig. 6). Microagglutination titers developed by day 7 following vaccination, remained constant for an additional 3 weeks and rose substantially following administration of the booster vaccination.

Immunodiffusion analysis. A titer produced against Washington whole cells or subunit preparations formed precipitin lines of identity

against all Washington antigens. These antisera also demonstrated identity between a minor component in Philadelphia L-1 and Washington antigen preparations. With antiserum against Washington ribosomes one of the precipitin lines revealed the presence of an antigen in Washington whole cell and ribosome preparations that was lacking in the Washington cell wall preparation. Antiserum against cell walls formed several lines of identity with all test antigens; one of the strongest reactions of the Washington antigens failed to appear with the Philadelphia L-1 antigens.

Antisera produced with Philadelphia L-1 whole cell, ribosome, and cell wall preparations also developed lines of identity against all Philadelphia L-1 antigens. No major precipitin reaction was observed between the Philadelphia L-1 whole cell antisera and Washington strain antigens. However, minor reactions were seen when antisera to Philadelphia L-1 subunits were reacted against Washington strain antigens.

DISCUSSION.

A procedure developed by Salton and Horne (25) for preparation of bacterial cell walls utilizing mechanical disruption and differential centrifugation has led to the investigation of the immunologic properties of subcellular units. Subsequent efforts have been directed towards development of more effective vaccines, and characterizing the immunogens and the quality of the immune response elicited by cellular subunits. Initially, cell walls were utilized to identify the location of bacterial protective antigens. It was not until Youmans et al. (33, 35) isolated immunogenic particles from Mycobacterium tuberculosis that ribosomes were shown to be effective vaccines. Since their studies,

cellular fractions from many organisms (bacteria, yeasts, protozoa) have been isolated, with emphasis on ribosomal preparations.

In the present study, cell wall and ribosomal vaccines from L. pneumophila were prepared by differential centrifugation of mechanically disrupted organisms. This is the first known research to employ the fractionation of L. pneumophila for investigating the immunogenicity and protective efficacy of its subunit components. The cell wall fraction is seen in electron photomicrographs to be composed of bacterial cell walls devoid of cytoplasmic constituents. This material was subjected to sonic treatment to assure disruption of the cell walls and to increase the effectiveness of subsequent treatment with RNase. However, sonic treatment resulted in the appearance of aggregates thought to be formed from cell wall material by the action of high frequency sound waves (24, 25, 27). This is attested to by the fact that the sonically treated preparation gives a single homogenous band after isopycnic centrifugation on sucrose gradients. After treatment with RNase, the final cell wall fraction contained less than 2 μ g RNA/mg of protein.

The ribosomal fraction appears morphologically homogenous. However, its RNA to protein ratio of 0.29 is not equal to the theoretical ratio for these constituents in purified bacterial ribosomes (14, 34). The low ratio obtained with the ribosomal preparation from L. pneumophila indicates contamination with proteins bound to the ribosomes during the isolation procedures. Contamination with endotoxin is also suspected based upon the detection of KDO in the fraction, possibly as a result of nonspecific binding of endotoxin to nucleic acids (9). The apparent efficacy of the ribosomal fraction therefore, cannot be ascribed to the ribosomes themselves but may be due either to the proteins or endotoxin present in the fraction.

Of the two subunit preparations, vaccination with cell walls consistently provided better protection of AKR/J mice against an i.p. challenge with the virulent Washington strain. The protective efficacy of cell walls suggests that they contain the major immunizing antigens against the pathogenic effects of the toxin(s), (15). The homologous strain precipitin reactions formed by antisera for Washington strain fractions indicate a common determinant group in ribosomal and cell wall fractions.

The difference in protective effect between the i.p. and s.c. routes of vaccination may reflect a wider distribution of antigens to immunoresponsive cells. Serologic responses, as evidenced by titers of circulating antibody, indicate this difference between routes of vaccination with homologous antigens.

In contrast to antigens derived from the Washington strain, Philadelphia L-1 preparations were substantially less effective immunogens. In fact, both cell wall and ribosomal preparations inoculated s.c. failed to show any protective efficacy. Immunoprecipitin reactions reveal a common antigen among the Philadelphia L-1 preparations, but weak reactions with Washington preparations, indicating that the strains differ in major antigenic components.

Antibodies produced by L. pneumophila have been shown to confer passive immunity (15, 31) and to have opsonic (18) and bactericidal (28) activity. The role of cell-mediated immunity has not been explored, although cell-mediated immunity often develops in response to intracellular pathogens; L. pneumophila has been shown to survive and multiply intracellularly (16, 21, 32). Ribosomal vaccines have been

suggested as being effective against intracellular pathogens (7, 17, 19, 22), but no conclusions can be drawn at this time concerning their efficacy against L. pneumophila infection. The protection elicited by the Washington ribosomal vaccine could be due to the protein contamination of the fraction.

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TABLE 1. Percent distribution of biochemical constituents among subcellular fractions of *L. pneumophila* obtained during the preparation of cell wall and ribosome vaccine preparations

Fraction	% Protein	% CHO	% DNA	% RNA	% KDO
Supernatant I ^a	100	100	100	100	100
Pellet II	2.02 ± 1.31	1.62 ± 1.37	2.84 ± 1.37	2.83 ± 0.91	7.06 ± 2.79
Supernatant II	96.13 ± 7.30	85.54 ± 12.99	101.59 ± 8.95	93.76 ± 13.59	ND ^b
Pellet III	30.79 ± 9.83	19.46 ± 3.88	21.10 ± 5.71	30.85 ± 23.66	ND
Pellet III A	31.16 ± 9.69	18.41 ± 5.86	20.19 ± 6.91	32.17 ± 25.68	ND
Supernatant III	63.47 ± 8.40	72.66 ± 13.80	68.83 ± 27.55	83.79 ± 6.25	ND
Pellet IV	34.42 ± 5.44	9.27 ± 7.10	19.30 ± 9.60	3.30 ± 3.05	ND
Supernatant IV	6.31 ± 1.77	7.64 ± 3.21	4.85 ± 0.46	12.55 ± 2.62	ND
Pellet IV A	24.02 ± 0.96	8.57 ± 4.90	6.09 ± 5.42	0.70 ± 0.45	38.79 ± 3.96
Supernatant IV A	6.01 ± 1.36	7.77 ± 1.68	2.72 ± 3.13	0.60 ± 0.62	3.75 ± 3.06
Pellet V	13.45 ± 3.01	21.09 ± 17.56	12.39 ± 3.71	57.23 ± 8.06	11.62 ± 1.09
Supernatant V	43.27 ± 2.49	41.84 ± 36.98	71.39 ± 2.49	11.93 ± 10.81	18.71 ± 0.93
% Recovery ^c	88.77	80.89	95.42	73.29	79.93

^a Supernatant I contained 998.68, 69.01, 69.66, 67.86, and 6.47 mg of protein, CHO, DNA, RNA and KDO, respectively. Results are means ± SD of three individual experiments.

^b Not done.

^c Fractions measured for percent recovery include: Pellet II, Pellet IV A, Supernatant IV A, Pellet V + Supernatant V.

TABLE 2. Protection afforded AKR/J mice (n = 6/group) immunized
with cell wall and ribosome vaccines prepared from
L. pneumophila Washington and Philadelphia L-1 strains
and challenged i.p. with Washington strain

Immunization to challenge time	Protein dose (μ g)	Route	% Survival			
			Washington		Philadelphia	
			Ribosome	Cell wall	Ribosome	Cell wall
2 weeks	5	i.p.	17	100	ND ^a	ND
	50	i.p.	67	100		
	5	s.c.	67	50		
	50	s.c.	83	67		
4 weeks	5	i.p.	67	100	ND	ND
	50	i.p.	83	100		
	5	s.c.	17	83		
	50	s.c.	100	50		
10 days after booster at 4 weeks	0.05	i.p.	0	83	ND	ND
	0.5	i.p.	67	83		
	5	i.p.	100	100	17	83
	50	i.p.	100	100	50	100
	0.05	s.c.	0	0	ND	ND
	0.5	s.c.	0	17		
	5	s.c.	50	100	0	0
	50	s.c.	100	100	0	0
Challenge controls			(0/12) 0%		(1/6) 17%	

^aNot done.

TABLE 3. Microagglutination titers (5/group) and protection of
mice immunized with Washington or Philadelphia
cell wall or ribosome vaccine preparations and
challenged with Washington strain

Immunization	Microagglutination titer		% Protected (n = 6/group)
	Washington	Philadelphia	
None	< 1: 2	< 1: 2	6 (n = 18)
Washington ribosomes			
i.p.	1: 256	1: 128	100
s.c.	1: 64	1: 128	100
Washington cell walls			
i.p.	1:4056	1:2048	100
s.c.	1: 128	1: 128	100
Philadelphia ribosomes			
i.p.	1: 64	1:4096	50
s.c.	1: 16	1: 512	0
Philadelphia cell walls			
i.p.	1: 64	1:8192	100
s.c.	1: 16	1:2048	0

FIGURE LEGENDS

FIG. 1. Schematic diagram for isolation of cell wall and ribosome fractions from disrupted L. pneumophila.

FIG. 2. Effect of mechanical disruption of L. pneumophila Washington strain suspension on turbidity (O) and protein solubilization (⊗). Combined 0.5 N NaOH and 0.05% SDS results are shown by horizontal broken lines. Results are averages of three experiments.

FIG. 3. Electron micrography of L. pneumophila Washington strain, X 30,000. (A) Whole organisms, (B) after mechanical disruption, (C) Pellet III, crude cell wall fraction, and (D) Pellet IV, final cell wall fraction.

FIG. 4. Normalized equilibrium density distribution on sucrose gradients of crude cell wall fraction (top) and final cell wall fraction (bottom). ..

FIG. 5. Electron micrograph of Pellet V, ribosomal fraction of L. pneumophila Washington strain, X 30,000.

FIG. 6. Microagglutination titers of AKR/J mice immunized with Washington strain cell wall (O) or ribosome (⊗) vaccine preparations, 50 µg protein in 1 ml i.p. Five mice/day bled and sera pooled.











